Bioavailability of Black Tea Theaflavins: Absorption, Metabolism and Colonic Catabolism

Journal:	*Journal of Agricultural and Food Chemistry*
Manuscript ID	jf-2017-01707t.R2
Manuscript Type:	Article
Date Submitted by the Author:	07-Jun-2017
Complete List of Authors:	Pereira-Caro, Gema; Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA), Department of Food and Health Moreno Rojas, Jose-Manuel; IFAPA, Postharvest, technology and Food Industries Brindani, Nicolettaa; University of Parma, Department of Food and Drug Del Rio, Daniele; University of Parma, Department of Public Health Lean, Michael; University of Glasgow, University of Glasgow College of Medical, Veterinary and Life Sciences Hara, Yukihiko ; 1-18-15-510, Taihei Crozier, Alan; University of California, Nutrition

Bioavailability of Black Tea Theaflavins: Absorption, Metabolism and Colonic Catabolism

Gema Pereira-Caro,† José Manuel Moreno-Rojas,† Nicoletta Brindani, Daniele Del Rio, Michael E. J. Lean,§ Yuihiko Hara,¶ and Alan Crozier¥*

†Department of Food and Health, Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA), Avenida Menendez-Pidal, SN, 14004, Córdoba, Spain.

Department of Food and Drug, University of Parma, 43124 Parma, Italy

§ University of Glasgow College of Medical, Veterinary and Life Sciences, New Lister Building, Glasgow G31 2ER, UK

¶1-18-15-510, Taihei, Sumida-Ku, Tokyo, 130-0012 Japan

¥Department of Nutrition, University of California, Davis, California 95616-5270, USA

*Corresponding author: Phone: +1 530-746-1421;E-mail: alan.crozier44@gmail.com

ABSTRACT: Data obtained with in vitro fecal incubations and a feeding study indicate black tea theaflavin and its galloyl derivatives are not absorbed in detectable amounts in either the upper or the lower gastrointestinal tract. The theaflavin skeleton is comparatively resistant to degradation by colonic bacteria with a 67% recovery being obtained after a 24 h incubation which yielded 21 phenolic and aromatic catabolites. The theaflavin galloyl moiety was removed by the microbiota and the released gallic acid further transformed to 3-*O*-and 4-*O*-methyl gallic acids, pyrogallol-1-sulfate and pyrogallol-2-sulfate, which were excreted in urine in amounts equivalent to 94% of intake. The main urinary product potentially derived from breakdown of the theaflavin skeleton was 3-(4′-hydroxyphenyl)propionic acid. A number of the colonic catabolites originating from gallic acid and theaflavins have been reported to be bioactive in ex vivo and in vitro models with a variety of potential modes of action.

KEYWORDS: black tea theaflavins, bioavailability, colonic catabolism, phenolic catabolites, HPLC-HRMS

INTRODUCTION

Camellia sinensis-based teas, are widely consumed throughout the world,[1] and contain an array of flavan-3-ols and derived compounds.[2] Green teas are a rich mixture of flavan-3-ol monomers including (–)-epicatechin **1**, (+)-gallocatechin-3-*O*-gallate **2**, (–)-epicatechin-3-*O*-gallate **3** and (–)-epigallocatechin-3-*O*-gallate **4** (Figure 1). Black teas, produced by fermentation of green tea leaves, contain substantially reduced amounts of flavan-3-ol monomers as a result of polyphenol peroxidase- and oxidase-mediated conversion to theaflavins and more substantial amounts of thearubigins.[2-4] Thearubigins are an extremely complex mixture of high molecular weight polymers[5,6] while theaflavins are dimer-like structures in the form of theaflavin **5**, theaflavin-3-*O*-gallate **6**, theaflavin-3´-*O*-gallate **7** and theaflavin-3,3´-*O*-digallate **8** (Figure 1). These compounds are potential bioactive components in black tea, consumption of which has been linked to beneficial effects on health.[7,8] Animal and in vitro models have demonstrated that theaflavins (i) inhibit pro-inflamatory cytokines including tumour necrosis factor (TNF)-α and interleukins (IL)-1β and IL-6,[9] (ii) prevent of DNA damage in lymphocytes,[10] (iii) supress the formation of reactive oxygen species by preserving the activities of antioxidant enzymes and (iv) inhibit the formation of thiobarbituric acid reactive substances and copper-induced lipid peroxidation of LDL and HDL.[11] Human clinical trials have shown that long-term regular consumption of black tea can improve cardiovascular function by lowering blood pressure,[12] and reducing platelet activation and plasma C-reactive protein in healthy men.[13] In addition, prospective studies have shown an association between black tea consumption and the incidence of different types of cancers.[14-16]

However, there is very limited information about the potential protective effects of theaflavins and thearubigins in the large intestine, and the impact on health of any colonic microbiota-derived catabolites after they enter the bloodstream and are transported to other sites within the body.[17] Although consumed far more extensively in Europe and the United States than green tea, the absorption of (poly)phenols from black tea beverages of (poly)phenols, their human metabolism, and gut flora catabolism have received only limited attention. Such studies have focused on the

absorption of flavan-3-ol monomers[18] and flavonol glycosides[19,20] from black tea, with or without added milk which appeared to have relatively little effect. To date, there is one report on the absorption of trace amounts of theaflavins by humans equivalent to 0.001% of intake.[21] More recently, two studies have shown the importance of the colonic microbiota in the metabolism of theaflavins by both mice and humans.[22,23] The present study investigated metabolites and colonic catabolites excreted in urine after ingestion of 1 g of a theaflavin extract by two healthy volunteers. In addition, the theaflavin extract was incubated under anaerobic conditions with fresh human fecal samples from three donors. In both studies, identification and quantitation of theaflavins and their derived products used high-performance liquid chromatography-high resolution mass spectrometry (HPLC-HRMS).

MATERIAL AND METHODS

Chemicals and Reagents. Formic acid, 2′,4′,5′-trimethoxycinnamic acid, ethyl gallate, (–)-epicatechin, (+)-gallocatechin-3-*O*-gallate, (–)-epicatechin-3-*O*-gallate and (–)-epigallocatechin-3-*O*-gallate and the phenolics 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, ferulic acid, isoferulic acid, 4′-hydroxyphenylacetic acid, 3′-hydroxyphenylacetic acid, 3′-methoxy-4′-hydroxyphenylacetic acid (homovanillic acid), 3,4-dihydroxybenzoic acid (protocatechuic acid), 3-(3′-methoxy-4′-hydroxy)mandelic acid, 4′-hydroxymandelic acid, 3-(3′,4′-dihydroxyphenyl)propionic acid (dihydrocaffeic acid), 3-(3′,4′-dihydroxyphenyl)acetic acid (homoprotocatechuic acid), 3-(4′- hydroxyphenyl)propionic acid, phenylacetic acid, 1,3,5-trihydroxybenzene (phloroglucinol), 1,2,3-trihydroxybenzene (pyrogallol) and 1,2-dihydroxybenzene (catechol) were purchased from Sigma-Aldrich (Poole, Dorset, UK). 3′-Hydroxyhippuric and 3-*O*-methyl gallic acid were, respectively, purchased from Toronto Research Chemicals (Toronto, ON, Canada) and Extrasynthese (Genay, France). Caffeic acid-3′-sulfate, ferulic acid-4′-*O*-glucuronide, ferulic acid-4′-sulfate, 3-(4′-hydroxyphenyl)propionic acid-3'-*O*-glucuronide (dihydrocaffeic acid-3'-*O*-glucuronide),

3-(3′-hydroxyphenyl)propionic acid-4′-sulfate (dihydrocaffeic acid-4'-sulfate), 3-(4′-hydroxyphenyl)propionic acid-3′-sulfate (dihydrocaffeic acid-3'-sulfate), 3-(3′-methoxyphenyl)propionic acid-4′-*O*-glucuronide (dihydroferulic acid-4′-*O*-glucuronide) and 3-(3′-methoxyphenyl)propionic-acid-4′-sulfate (dihydroferulic acid-4′-sulfate) were kindly provided by Denis Barron (Nestle Research Center, Lausanne, Switzerland) and Gary Williamson (School of Food Science and Nutrition, University of Leeds, UK). 4′-Hydroxyhippuric acid was obtained from Bachem (UK) Ltd (St Helens, UK). Synthetic urine (Negative Urine Control) was purchased from Sigma-Aldrich, Madrid, Spain. 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone, 5-(3′-hydroxyphenyl)-γ-valerolactone and 5-(phenyl)-γ-valerolactone-3′-*O*-sulfate were synthesized according to Curti et al.[24] Reagents and chemicals used in the preparation of buffers employed in the fecal fermentation experiments were of analytical grade and purchased from Fisher Scientific (Loughborough, UK), Across Organics (Geel, Belgium), AnalaR (Pool, UK) and Sigma-Aldrich (Steinheim, Germany). HPLC-MS grade methanol and acetonitrile were obtained from Sigma-Aldrich (Steinheim, Germany).

Theaflavin Extract. The theaflavin extract was prepared commercially from an aqueous extract of fermented green tea leaves which, after membrane filtration, was fractionated by column chromatography using a macroporous resin before being spray dried to form a powder.

Human Feeding Study. Two healthy male volunteers between 29 and 33 years old with a BMI of 23.9 and 28.2 kg/m^2 were recruited at the University of Glasgow. The volunteers, who were non-smokers and not receiving medication, were informed about the aim of the study and gave their written consent before their particpation in the trial. Volunteers followed a low-(poly)phenol diet for 60 h prior to the study and for 30 h after supplementation, avoiding fruits, vegetables, high-fibre products, and beverages such as tea, coffee, fruit juice, and wine. Before feeding, the subjects provided 0-30 h baseline urine over four time periods (0-4, 4-12, 12-24 and 24-30 h). On the day of the feeding, after an overnight fast, each subject ingested 1 g of encapsulated theaflavin powder. A light breakfast of white bread, cheese, ham and milk was provided 2 h after the theaflavin intake.

Following supplementation all urine was collected over four time periods (0-4, 4-12, 12-24 and 24-30 h). The volume of urine excreted was measured and aliquots stored at -80 °C prior to HPLC-HRMS analysis. The study was approved by the University of Glasgow Royal Infirmary Ethics Committee.

Processing of Urine. Urine samples were prepared as follows: 4 mL of urine was added to 2 mL of 1% aqueous formic acid containing 2.8 μg of ethyl gallate as an internal standard. 4 mL of urine was added to 2 mL of 1% aqueous formic acid containing 2.8 μg of ethyl gallate as an internal standard. Strata-X (33 μ, 500 mg) solid phase extraction cartridges (Phenomenex, Macclesfield, U.K.) were washed and activated with 6 mL of MeOH and conditioned with 6 mL of H_2O/formic acid (99:1, v/v). The acidified urine was loaded onto the cartridge which was washed with 6 mL of H_2O/formic acid (99:1, v/v) before being eluted with 6 mL of MeOH/formic acid (99:1, v/v). The eluate was reduced to dryness in vacuo in a Speed Vac protected from light at 35 °C and re-suspended in 50 μL of MeOH/formic acid (99:1, v/v) and 150 μL of deionized water.

Fecal Sample Preparation. Fecal samples were obtained from three non-smoker volunteers (two female and one male, different individuals to those who participated in the feeding study), who had not consumed antibiotics for at least 3 months before the study. The volunteers followed a (poly)phenol-low diet for 48 h before fecal sample collection. The collection of the fecal samples and the preparation of the fecal slurries was carried out according to Roowi et al. [25]

Fermentation Medium and fecal incubation. Fermentation medium was prepared according to Roowi et al. [25] The fecal incubation used procedures described by Pereira-Caro et al. [26] The theaflavin extract was added to a final concentration of 10 μmol per 50 mL of incubation medium. Aliquots (5 mL) of fecal suspensions were taken after 0 h, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h and stored immediately at –80 °C.

Extraction of Theaflavins from Fecal Incubates. Extraction of theaflavins from faecal incubates was adapted from Pereira-Caro et al.[26] with some modifications. Briefly, 500 μL of defrosted fecal sample was spiked with 300 μg of 2′,4′,5′-trimethoxycinnamic acid, used as internal

standard. The supernatants obtained after three ethyl acetate extractions were reduced to dryness in vacuo and resuspended in 1 mL MeOH/water/formic acid (49.5:49.5:1, v/v/v).

Analysis of Theaflavins and Their Metabolites in Urine and Fecal Samples. The theaflavin extract, SPE-concentrated urine samples and purified fecal samples were analysed using a Dionex Ultimate 3000 UHPLC system comprising of a UHPLC pump, a PDA detector scanning from 200 to 600 nm, and an autosampler operating at 4° C (Thermo Scientific, San Jose, CA, USA). A 150 x 4.6 mm i.d. 2.6 µm 100A XB-C18 Kinetex column (Phenomenex, Macclesfield, UK) was used for the analysis of the theaflavins, and a 150 x 4.6 mm i.d. 5 µm 100 A C18 Kinetex column (Phenomenex) for the analysis of the phenolic and aromatic catabolites, (−)-epicatechin metabolites and phenylvalerolactones and valeric acids. Both columns were maintained at 40 °C and eluted at a flow rate of 1.0 mL/min, with, respectively, a 70 min gradient of 5-40% acetonitrile in 0.1% aqueous formic acid, and a 42 min gradient of 3-50% methanol in 0.1% aqueous formic acid. After passing through the flow cell of the PDA detector the column eluate was split and 0.2 mL/min directed to an Exactive Orbitrap mass spectrometer (Thermo Scientific, San José, CA, USA) fitted with a heated electrospray ionization probe (HESI) operating in negative ionization mode. Full scans were recorded in a *m/z* range of 100-1800 with a resolution of 50.000 and with a full AGC target of 1000000 charges, using 2 microscans. Analyses were also based on scans with in-source collision-induced dissociation at 25.0 eV. Two MS conditions with HESI in negative ionization mode were used: (i) capillary temperature was 300 °C, the heater temperature was 150 °C, the sheath gas was 60 units, the auxiliary gas was 20 units, and the spray voltage was 4.0 kv for the analysis of theaflavins; (ii) the capillary temperature was 275 °C, the heater temperature was 150 °C, the sheath gas was 20 units, the auxiliary gas was 16 units, and the spray voltage was 4.0 kv for the analysis of phenolic catabolites, epicatechin metabolites and phenylvalerolactones and valeric acids. Data acquisition and processing were carried out using Xcalibur 3.0 software (Thermo Scientific, San José, CA, USA). Before a measurement series, the Exactive Orbitrap was calibrated externally on a weekly basis using a ready-to-use calibration mixture (Pierce ESI Negative Ion Calibration Solution),

obtained from Thermo Scientific. Targeted identifications of theaflavins and their potential metabolites and catabolites were achieved by co-chromatography and comparing the exact mass with available standards. When standards were not available, compounds were tentatively identified by comparing the theoretical exact mass of the molecular ion with the measured accurate mass of the molecular ion. Identifications were categorized according to the Metabolite Standards Initiative Metabolite Identification (MSIMI) levels.[27]

Theaflavins were quantified based on chromatographic 365 nm peak areas and expressed relative to standards of theaflavins. Quantification of metabolites and catabolites were carried out by selecting the theoretical exact mass of the molecular ion by reference to 0.05-150 ng standard curves. The linearity was determined for all the available standards. Limits of detection (LOD) and limits of quantification (LOQ) were estimated from the signal to noise ratio of each compound showing a signal-to-noise ratio ≥ 3 and ≥ 10, respectively. LOD and LOQ ranged from 0.03 to 0.1 ng and from 0.09 to 0.7 ng, respectively. The precision of the assay (the coefficient of intra-assay variation) was ranged from 1.1 to 5.2%. All reference compounds used for calibration curves were made up in synthetic urine and a blank fecal extract processed as described above. In absence of reference compounds, metabolites were quantified by reference to the calibration curve of a closely related parent compound.

Statistical Analysis. Because of the sample size, Wilcoxon post-hoc signed-rank test was used to determine whether differences in total excretion of metabolites and phenolic catabolites after theaflavin intake. Significance was accepted at the $P<0.05$ level and data are presented as mean values ± SEM unless stated otherwise. All statistical analyses were performed using the Statistix 9.0 software.

RESULTS AND DISCUSSION

Characterization of the Theaflavin Extract. HPLC-HRMS analysis of the theaflavin extract

revealed the presence of four individual theaflavins. The quantities of each individual theaflavin, along with the small amounts of (–)-epicatechin **1**, (+)-gallocatechin-3-*O*-gallate **2**, (–)-epicatechin-3-*O*-gallate **3**, (–)-epigallocatechin-3-*O*-gallate **4** and procyanidin dimer B2-3′-*O*-gallate **9** (Figure 1), which were present in 1 g of the extract are shown in Table 1. When consuming 1 g of the extract the volunteers ingested 177 μmol, 307 μmol, 172 μmol and 332 μmol of theaflavin **5**, theaflavin-3-*O*-gallate **6**, theaflavin-3'-*O*-gallate **7** and theaflavin-3,3'-*O*-digallate **8**, respectively, a total of 988 μmol. The ingested 1014 μmol supplement, therefore, contained >97% theaflavins, together with 3.4 μmol of flavan-3-ol monomers and 22.7 μmol of procyanidin dimer B2-3′-*O*-gallate **9**.

Urinary Excretion of Theaflavins and Metabolites. No theaflavins **5**, theaflavin-3-*O*-gallate **6**, theaflavin-3'-*O*-gallate **7** or theaflavin-3,3'-*O*-digallate **8** or their phase II metabolites were detected in urine excreted 0-30 h after an intake of the 1 g theaflavin extract by the two volunteers.

The four flavan-3-ols monomers (Table 1) were following ingestion, in part, be excreted in urine as glucuronide, sulfate and methylated metabolites, referred to as structurally-related epicatechin metabolites (SREMs).[28] The 3.4 μmol of monomers, along with the 22.7 μmol of procyanidin dimer B2-3′-*O*-gallate **9** in the extract, also underwent undergo a partial conversion to 5C-ring fission metabolites (5C-RFMs), the phenyl-γ-valerolactones and the phenyl-γ-hydroxyvaleric acids and their phase II metabolites[28,29]. No SREMs or 5C-RFMs were excreted in urine pre-supplementation and the amounts excreted over the 0-30 h collection period after acute ingestion of the theaflavin extract are presented in Table 2. Seven SREMs were detected in amounts ranging from 0.9 ± 0.2 nmol for an epicatechin-*O*-glucuronide to 43 ± 8 nmol for an *O*-methyl-(–)-epicatechin-*O*-glucuronide. Seventeen 5C-RFMs in the form of sulfates and glucuronide conjugates of phenyl-γ-valerolactones and phenyl-γ-hydroxyvaleric acids were identified and quantified. Overall 60 ± 15 nmol of SREMs were excreted along with 1437 ± 436 nmol of 5C-RFMs (Table 2)

Urinary Phenolic and Aromatic Catabolites. HPLC-HRMS analysis revealed a total of 41

phenolic and aromatic compounds excreted in urine 0-30 h following an acute intake of 1 g of theaflavins (Table 3). Unlike the SREMs and 5C-RFMs, many of these compounds are produced in the body by pathways unrelated to (poly)phenol intake[2]. Hence, in order to assess the impact of theaflavin consumption, the quantitative data in Table 3 are adjusted values obtained by subtraction of the amount of phenolics excreted in the 30 h pre-supplementation period. Twenty one metabolites were excreted in significantly increased amounts. They were 3-(4′-hydroxyphenyl)propionic acid **10**, 3′,4′-dihydroxyphenylacetic acid **11**, 3′-methoxy-4′-hydroxyphenylacetic acid **12**, 3′-hydroxyphenylacetic acid **13**, 4′-hydroxyphenylacetic acid **14**, phenylacetic acid **15**, 3,4-dihydroxybenzoic acid **16**, 3-hydroxybenzoic acid **17**, 4-hydroxybenzoic acid **18**, benzoic acid-4-sulfate **19**, 3-*O*-methylgallic acid **20**, 4-*O*-methylgallic acid **21**, gallic acid **22**, phloroglucinol (1,3,5-trihydroxybenzene) **23**, catechol (1,2-dihydroxybenzene) **24**, pyrogallol (1,2,3-trihydroxybenzene) **25**, pyrogallol-1-*O*-sulfate **26,** pyrogallol-2-*O*-sulfate **27**, pyrogallol-2-*O*-glucuronide **28**, 3′-hydroxyhippuric acid **29** and 4′-hydroxyhippuric acid **30** (Figure 2). The main increase was in the excretion of hydroxybenzene derivatives (Table 3). Many of these compounds have previously been detected in urine after ingestion of black tea[30-33]. In total the increased excretion of the 21 catabolites was 955 ± 376 μmol equivalent to 94% of the 1014 μmol intake of polyphenolic compounds (Table 3)

Theaflavin Degradation by Fecal Suspensions. Fecal samples from three donors, which HPLC-HRMS showed did not contain detectable quantities of theaflavins when voided, were incubated in vitro under anaerobic conditions with 10 μmol of the theaflavin extract for a period of 24 h and aliquots taken over a range of time points were analyzed by HPLC-HRMS. Figure 3 illustrates the levels of the four individual theaflavins in the incubates. With samples from volunteers 1 and 2 incubation led to an increase in theaflavin **5** and a concomitant decline in the levels of theaflavin-3-*O*-gallate **6**, theaflavin-3'-*O*-gallate **7** and theaflavin-3,3'-*O*-digallate **8**, a consequence of cleavage of the 3 and 3'-*O*-gallate moieties. These changes were less evident in incubations with feces from volunteers 3 seemingly as a consequence of reduce degalloylation by the

fecal bacteria of this donor. At the end of the 24 h incubation period the total theaflavins remaining, from the 10 μmol added at 0 h, were 7.5 μmol (volunteer 1), 6.7 μmol (volunteer 2) and 6.0 μmol (volunteer 3) (Table 4) giving an average value of 6.7 ± 0.4 μmol, corresponding to a 67% recovery.

The quantities of phenolic catabolites formed during the incubation of the theaflavin extract with the fecal samples are also presented in Table 5. The values are corrected for baseline levels of benzoic acid, and 3-(phenyl)propionic and phenylacetic acids present in the control fecal media. The fermentations resulted in the appearance of relatively small amounts of 6 phenolic catabolites, with 3-hydroxybenzoic acid **17**, pyrogallol **25**, and benzoic acid **31** as the major transformation products after 8 h and 24 h incubations in samples from donors 1 and 2. Low levels of gallic acid **22** were detected in the first 1 h of incubation of feces from two donors, the levels of which declined completely after 24 h of incubation (Table 5). This decline was accompanied by appearance of pyrogallol **25** in the 6-24 h of incubation. In addition, low levels of 3,4-dihydroxybenzoic acid **16** and 3-(3,4´dihydroxyphenyl)propionic acid **32** were detected after 2, 4, 6 and 8 h although not in all samples from the 3 donors. Indeed, there were substantial differences in the catabolite profiles of the 3 donors, presumably due to inter-individual variations in the colonic microbiota.

Other catabolites such as 5-(3´-hydroxyphenyl)-ɣ-hydroxyvaleric acid **33**, 5-(phenyl)-ɣ-hydroxyvaleric acid **34** and 5-(3´,4´-dihydroxyphenyl)- ɣ-valerolactone **35** accumulated in small amounts, while 5-(3´-hydroxyphenyl)-ɣ-valerolactone **36** appeared transitorily after 8 h of incubation (Table 5). These four 5C-RFMs are known colonic degradation products of flavan-3-ols monomers and possibly to some extent the procyanidin dimer[28,29] a small quantity of which was present in the theaflavin extract (Table 1).

Although a preliminary study because of the limited number of volunteers involved, this detailed investigation none-the-less provided much novel information on the fate of black tea theaflavins following ingestion. Mulder et al.[21] previously reported the presence of trace amounts of theaflavins in plasma and urine, corresponding to 0.0006% of intake after the ingestion of a 700 mg mixture of theaflavins by two volunteers. Although quantities will vary from tea to tea depending

mainly upon the tea leaves and the degree to which they were fermented, based on the data of Del Rio et al.[3] this is also equivalent to ~10 cups of tea. In the present study, after ingestion of a 1 g supplement containing 998 μmol of a mixture of theaflavins (Table 1), also equivalent to ~10 cups of tea, no theaflavins were detected in urine. The HPLC-HRMS limit of detection for theaflavin reference compounds was 0.3 ng. Taking into account that the urine samples were concentrated 20-fold prior to analysis using Strata X solid phase extraction cartridges, total urinary theaflavin excretion 0-30 h after intake was <6 ng, representing <0.000001 % of intake. These results confirm, using much higher doses, the findings of Henning et al.[34] who reported an absence of theaflavins in urine and prostate tissue from men who consumed six cups of black tea daily for a period of 3-8 weeks.

The evidence, therefore, indicates that black tea theaflavins have poor systemic bioavailability. During passage along the GI tract, substantial amounts will reach the colon where they will be subject to the action of the resident microbiota, potentially resulting in the formation of low molecular weight degradation products. In keeping with this possibility, after ingestion of the theaflavin extract in the current study, increased amounts of phenolic catabolites, 964 μmol, were detected in 0-30 h urine, corresponding to 94% of the 1014 μmol intake of polyphenols which was composed predominantly, of 988 μmol, of theaflavins (Table 3). However, it does not necessarily follow that this is the result of an extensive breakdown of the basic theaflavin ring structure. Most of the increased excretion of phenolics was due to enhanced quantities of hydroxybenzene derivatives, in particular 625 μmol of pyrogallol-1-sulfate **26** and pyrogallol-2-sulfate **27** (Table 3). These catabolites were probably derived from gallic acid **22** released by hydrolysis of the gallate moiety of the 811 μmol, of the ingested gallated theaflavins. The other phenolic compound to be excreted in a substantially increased amount after theaflavin consumption was the 166 μmol of 3-(4'-hydroxyphenyl)propionic acid **10** (Table 3). This phenolic acid probably originated from theaflavin rather than gallic acid breakdown with much smaller amounts arising from ring fission of the 26 μmol of flavan-3-ol monomers and procyanidin dimer in the theaflavin extract.

A large number of phenolic catabolites have previously been identified following feeds[20, 25, 30-35,] and in vitro fecal incubations with black tea.[36,37] However as black tea contains numerous (poly)phenolics and related compounds,[2] these studies unlike the current investigation, were unable to identify catabolites arising from theaflavins.

Evidence for the stability of theaflavins in the lower gastrointestinal tract was obtained from the fecal incubations where the galloyl group was cleaved from theaflavin-3-*O*-gallate **6**, theaflavin-3'-*O*-gallate **7** and theaflavin-3,3'-*O*-digallate **8**. The released theaflavin skeleton **5** was relatively resistant to degradation with a 67% overall recovery of the theaflavins. This greatly exceeds the recoveries of flavan-3-ol monomers, flavanones and other dietary (poly)phenols when they are subjected to fecal incubations.[25,26,38]

A total of 60 nmol of SREMs were excreted in urine. These would originate from the 3.4 μmol of flavanol monomers in the theaflavin extract (Table 1) while the 1437 nmol excretion of 5C-RFMs are also likely to be derived from the monomers and to some degree the procyanidin dimer, combined intake of which was 26 μmol. This represents a 5.7% conversion.

There is a report of an increase in urinary excretion of hippuric acid **37** after black tea consumption by habitual tea drinkers.[35] However, this increase is unlikely to be a consequence of microbial degradation of theaflavins, as no increase in the urinary excretion of hippuric acid was observed in the present study following ingestion of theaflavins in an amount equivalent to ~10 cups of black tea. The 9.4 μmol increase in urinary excretion of 3′-hydroxyhippuric acid **29** and 4′-hydroxyhippuric acid **30** could to some degree be associated with theaflavin **5** degradation although a very minor portion of the increase in 3′-hydroxyhippuric acid could be related to flavan-3-ol monomer and procyanidin breakdown[28,29].

The principle routes for the limited degradation of theaflavins may involve the proposed pathways, illustrated in Figure 4. The fecal incubations reveal that microbial enzymes result in the release of substantial amounts of gallic acid **22** from the galloylated-theaflavins. In vivo some of the

gallic acid **22** then appears to be converted to 3-*O*-methygallic acid **20** and 4-*O*-methygallic acid **21** by mammalian enzymes while most is decarboxylated, probably by bacterial enzymes, to form pyrogallol **25** which undergoes phase II metabolism yielding substantial amounts of pyrogallol-1-sulfate **26** and pyrogallol-2-sulfate **27** and smaller amounts of a pyrogallol-2-*O*-glucuronide **28** (Table 3, Figure 4). The fecal incubations indicate that gallic acid 2**2** can also be dehydroxylated yielding in turn 3,4-dihydroxybenzoic acid **16** and 3-hydroxybenzoic acid **17** (Table 5) with the latter potentially being glycinated in vivo by hepatic enzymes to form 3′-hydroxyhippuric acid **29**.

Urinary excretion suggests that the limited degradation of theaflavin **5** appears to be linked to a plethora of minor products the major component of which is 3-(4′-hydroxyphenyl)propionic acid **10**. Its formation would appear to involve mammalian as well as bacterial enzymes as it was not produced by the fecal incubates although small amounts of 3-(3′,4′-dihydroxyphenyl)propionic acid **32** did accumulate (Table 5). Potentially, further metabolism of 3-(4′-hydroxyphenyl)propionic acid **10** could involve side chain shortening via β-oxidation producing 4-hydroxybenzoic acid **18**, a substrate for possible glycination in the liver to form 4′-hydroxyhippuric acid **30** (Figure 4). The, no doubt, complex array of pathways leading from theaflavin **5** to trace levels of the array of other phenolic catabolites, such as 3′-hydroxyphenylacetic acid **13,** 4′-hydroxyphenylacetic acid **14** and 3′-methoxy-4′-hydroxymandelic acid **38** (Table 3), is very much a matter of speculation and their elucidation represents a major analytical task. Some clarification could be achieved by carrying out separate fecal incubations with gallic acid **22** and non-galloylated theaflavins **5**.

The data obtained in the present study suggest that beneficial effects associated with theaflavins arising from consumption of black tea would, most likely, be derived from their circulating metabolites/catabolites which are mainly of microbial origin. Two of the microbial bioconversion products, 3,4-dihydroxybenzoic acid **16** and phloroglucinol **23**, along with their phase II sulfate and glucuronide metabolites, have been reported to reduce the levels of pro-inflammatory chemokines and adhesion molecules, such as IL-6 and sVCAM-1, in CD40L and oxidized

LDL-challenged vascular endothelial cells,[39] TNF-α secretion in THP-1 monocytes at physiologically relevant concentrations,[40] and sVCAM-1 secretion by human umbilical vein endothelial cells.[41] It has also been reported that 3-(4´-hydroxyphenyl)propionic acid **10** and gallic acid **22**, two key theaflavin-derived colonic catabolites, are able to protect neuronal cells from oxidative stress working as active agents against the onset and progress of neurodegenerative diseases.[42] Gallic acid **22** also has protective effects on multiple parameters involved in atherosclerosis, including inflammation, cell adhesion, chemotaxis, endothelial function, estrogenic/antiestrogenic activity and angiotensin-converting enzyme inhibitory activity[43] and on the inhibition of cell proliferation and induction of apoptosis in human colon cancer cells.[44] Furthermore, 3-*O*-methylgallic acid **20** and 4-*O*-methygallic acid **21**, two biomarkers of theaflavin intake, have been reported to exert protective effects in vitro, at physiological concentrations. 3-*O*-Methylgallic acid **20** has been shown to cause a time- and dose-dependent decrease in cell viability, cell cycle arrest at G_0/G_1 and to stimulate the apoptotic pathway by the activation of caspase-3 in human colon cancer cells, thereby acting as anticancer agent,[44] while 4-*O*-methylgallic acid **21** exhibited anti-inflammatory properties playing a role in the prevention of atherosclerotic processes[45] and the treatment of endotoxemia.[46] In addition, pyrogallol **25** has been reported to counteract key features of diabetes in vitro.[47]

AUTHOR INFORMATION

Corresponding Author

*Phone: 530-754-0450: email: alan.crozier44@gmail.com

Funding

GP-C was supported by a postdoctoral research contract “Juan de la Cierva-Incorporación” funded by the Spanish Ministry of Economy and Competitiveness (FJCI-201526433) and IFAPA.

Notes

AC is a consultant for Mars Inc. and has received unrestricted research grants from Mars and

research grants from other food companies and government agencies with an interest in health and nutrition. The other authors declare no conflict of interest

ACKNOWLEDGEMENTS

We thanks the volunteers who participated in the feeding study and who provided fecal samples.

ABBREVIATIONS USED

HPLC-HRMS: high-performance liquid chromatography-high resolution mass spectrometry; TNF, tumour necrosis factor; IL, interleukin; oxygen-free nitrogen, ONF.

SUPPORTING INFORMATION

Table S1. HPLC-HRMS-based identifications of structurally-related epicatechin metabolites (SREMs), and the 5 carbon side chain ring fission metabolites (5C-RFMs) phenyl-γ-valerolactones and phenyl-γ-hydroxyvaleric acids in human urine collected 0-30 h after theaflavin consumption and after in vitro fecal fermentation of the theaflavin extract.

Table S2. HPLC-HRMS-based identifications of phenolic catabolites in human urine collected 0-30 h after theaflavin consumption and in fecal samples after in vitro fermentation of the theaflavin extract.

This material is available free of charge via the Internet at http:pubs.acs.org.

REFERENCES

(1) Bond, T. J. The origins of tea, coffee and cocoa as beverages. In: Crozier A, Ashihara H, Tomás-Barberán F, eds. *Tea, Cocoa and Coffee: Plant Secondary Metabolites and Health*. Oxford, United Kingdom: Wiley-Blackwell Publishing, **2012**:1–24.

(2) Clifford, M. N.; Crozier, A. Phytochemicals in teas and tisanes and their bioavailability. In: Crozier A, Ashihara H, Tomás-Barberán F, eds. *Tea, Cocoa and Coffee: Plant Secondary Metabolites and Health.* Oxford, United Kingdom: Wiley-Blackwell Publishing, **2012**:45–98.

(3) Del Rio, D.; Stewart, A. J.; Mullen, W.; Burns, J.; Lean, M. E. J., Brighenti, F.; Crozier, A. HPLC-MS^n analysis of phenolic compounds and purine alkaloids in green and black tea. *J. Agric. Food Chem*. **2004**, *52*, 2807−2815.

(4) Li, S.; Lo, C.; Pan, M. H.; Lai, C. S.; Ho, C. T. Black tea: chemical analysis and stability. *Food Funct*. **2013**, 4: 10−18.

(5) Kuhnert, N. Unraveling the structure of the black tea thearubigins. *Arch. Biochem. Biophys*. **2010**, *50*, 37−51.

(6) Kuhnert, N.; Drynan, J. W.; Obuchowicz, J.; Clifford, M. N.; Witt, M. Mass spectrometry characterization of black tea thearubigins leading to an oxidative cascade hypothesis for thearubigin formation. *Rapid Commun. Mass Spectrom*. **2010**, *24*, 3387−3404.

(7) Blot, W. J.; McLaughlin, J. K.; Chow, W. H. Cancer rates among drinkers of black tea. *Crit. Rev. Food Sci. Nutr*. **1997**, *37*, 739−760.

(8) Sharma, V.; Rao, L. J. A thought on the biological activities of black tea. *Crit. Rev. Food Sci. Nutr.* **2009**, *49*, 379−404.

(9) Neyestani, T. R.; Gharavi, A.; Kalayi, A. Selective effects of tea extract and its phenolic compounds on human peripheral blood mononuclear cell cytokine secretions. *Int. J. Food Sci. Ntr.* **2009**, *60*, 79−88.

(10) Jezovicová, M.; Konarikova, K.; Durackova, Z.; Kerestes, J.; Králik, G.; Zitnanová, I. Protective effects of black tea extract against oxidative DNA damage in human lymphocytes. *Mol. Med. Reports.* **2016**, DOI: 10.3892/mmr.2015.4747

(11) Liu, S.; Huang, H. Assessments of antioxidant effect of black tea extract and its reationals by erithrocyte haemolysis assay, plasma oxidation assay and cellular antioxidant activity (CAA) assay. *J. Func. Foods* **2015**, *18*, 1095−1105.

(12) Hodgson, J. M.; Puddey, I. B.; Woodman, R. J.; Mulder, T. P.; Fuchs, D.; Scott, K.; Croft, K. D. Effects of black tea on blood pressure: a randomized controlled trial. *Arch. Intern. Med*. **2012**, *172*, 186−188.

(13) Steptoe, A.; Gibson E. L.; Vuononvirta, R.; Hamer, M.; Wardle, J.; Rycroft, J. A.; Martin, J. F.; Erusalimsky, J. D. The effects of chronic tea intake on platelet activation and inflammation: a double-blind placebo controlled trial. *Atherosclerosis* **2007**, *193*, 277–282.

(14) Baker, J. A.; Boakye, K.; McCann, S. E.; Beehler, G. P.; Rodabaugh, K.; Villella, J. A.; Moysich, K. B. Consumption of black tea or coffee and risk of ovarian cancer. *Int. J. Gynecol. Cancer* **2007**, *17*, 50–54.

(15) Wang, L.; Zhang, X.; Liu, J.; Shen, L.; Li, Z. Tea consumption and lung cancer risk: a meta-analysis of case-control and cohort studies. *Nutrition* **2014**, *30*, 1122–1127.

(16) Cassidy, A.; Huang, T.; Rice. M.S.; Rimm, E. B.; Tworoger, S. S. Intake of dietary flavonoids and risk of epithelial ovarian cancer. *Am. J. Clin. Nutr*. **2014**, *100*, 1344–1351.

(17) Clifford, M. N.; van der Hooft, J. J. J.; Crozier, A. Human studies on the absorption, distribution, metabolism and excretion of tea polyphenols. *Am. J. Clin. Nutr*. **2013**, *98*, 1619S–1630S.

(18) Henning, S. M.; Niu, Y.; Lee, N. H.; Thames, G. D.; Minutt, R. R.; Wang, H.; Go, V. L.; Heber, D. Bioavailability and antioxidant activity of tea flavanols after consumption of green tea, black tea, or a green tea extract supplement. *Am. J. Clin. Nutr*. **2004**, *80*, 1558–1564.

(19) de Vries, J. H.; Hollman, P. C.; Meyboom, S.; Buysman, M. N.; Zock, P. L.; van Staveren, W. A.; Katan, M. B. Plasma concentration and urinary excretion of the antioxidant flavonols queercetin and kaempferol as biomarkers for dietary intake. *Am J. Clin Nutr*. **1998**, *68,* 60–65.

(20) Hollman, P. C.; van het Hof, K. H.; Tijburg, L. B.; Katan, M. B. Addition of milk does not affect the absorption of flavonols from tea in man. *Free Radic. Res.* **2001**, *34*, 297–300.

(21) Mulder, T. P.; van Platerink, C. J.; Wijnand Schuyl, P. J.; van Amelsvoort, J. M. Analysis of theaflavins in biological fluids using liquid chromatography-electrospray mass spectrometry. *J. Chromatogr. B* **2001**, *760*, 271–279.

(22) Chen, H.; Hayek, S.; Guzman, J. R.; Gillitt, N. D.; Ibrahim, S. A.; Jobin, C.; Sang, S. The microbiota is essential for the generation of black tea theaflavins-derived metabolites. *PlosOne*. **2012**, 12, e51001-2012

(23) Chen, H.; Parks, T. A.; Chen, X.; Gillitt, N. D.; Jobin, C.; Sang, S. Structural identification of mouse fecal metabolites of theaflavin-3,3´-digallate using liquid chromatography tandem mass spectrometry. *J. Chromatogr. A.* **2011**, *1218*, 7297–7306.

(24) Curti, C.; Brindani, N.; Battistini, L.; Sartori, A.; Pelosi, G.; Mena, P.; Brighenti, F.; Zanardi, F.; Del Rio, D. Catalytic, enantioselective vinylogous Mukaiyama aldol reaction of furan-based dienoxy silanes: a chemodivergent approach to γ–valerolactone flavan-3-ol metabolites and δ-lactone analogues. *Adv. Syn. Catalysis* **2015**, *357*, 4082–4092.

(25) Roowi, S.; Stalmach, A.; Mullen, W.; Lean, M. E. J.; Edwards, C. A.; Crozier, A. Green tea flavan-3-ols: colonic degradation and urinary excretion of catabolites by humans. *J. Agric. Food Chem.* **2009**, *58*, 1296–1304.

(26) Pereira-Caro, G., Borges, G.; Ky, I.; Ribas, A.; Calani, L.; Del Rio, D.; Clifford, M. N.; Roberts, S. A.; Crozier, A. In vitro colonic catabolism of orange juice (poly)phenols. *Mol. Nutr. Food Res*. **2015**, *59*, 465–475.

(27) Summer, L. W.; Amberg, A.; Barrett, D.; Beale, M. H.; Beger, R.; Daykin, C. A.; Fan, T. W.; Fiehn, O.; Goldagre, R.; Griffin, J. L. Proposed minimum reporting standards for chemical analysis. Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics* **2007**, *3*, 211–221.

(28) Ottaviani, J. I.; Borges, G.; Momma, T.; Spencer, J. P. E.; Keen, C. L.; Crozier, A.; Schroeter, H. The metabolome of [2-^{14}C](–)-epicatechin in humans: implications for the assessment of efficacy, safety, and mechanism of action of polyphenolic bioactives. *Sci. Rep*. **2016**, *6*, 29034.

(29) Stoupi, S.; Williamson, G.; Dryna, J. W.; Barron, D.; Clifford, M. N. A comparison of the in vitro biotransformation of (–)-epicatechin and procyanidin B2 by human faecal bacteria. *Mol. Nutr. Food Res*. **2010**, *54*, 747–759.

(30) Daykin, C. A.; van Duynhoven, J. P.; Groenewegen, A.; Dachtler, M.; van Amelsvoort, J. M.; Mulder, T. P. Nuclear magnetic resonance spectroscopic based studies of the metabolism of black tea polyphenols in humans. *J. Agric. Food Chem*. **2005**, *53*, 1428–1434.

(31) van Dorsten, F. A.; Daykin, C.A.; Mulder, T.P.; Van Duynhoven, J.P. Metabolomics approach to determine metabolic differences between green tea and black tea consumption. *J. Agric. Food Chem*. **2006**, *54,* 6929–6938.

(32) van der Hooft, J. J. J.; Vos, R. C. H.; Mihaleva, V.; Bino, R. J.; Ridder, L.; de Roo, N.; Jacobs, D. M.; van Duynhoven, J. P. M.; Vervoort, J. Structural elucidation and quantification of phenolic conjugates present in human urine after tea intake. *Anal. Chem*. **2012**, *84*, 7263–7271.

(33) Hodgson, J. M.; Morton, L. W. M.; Puddey, I. B.; Beilin, L. J.; Croft, K. D. Gallic acid metabolites are markers of black tea intake in humans. *J. Agric. Food Chem*. **2000**, *48*, 2276–2280.

(34) Henning, S.M.; Wang, P.; Said, J.W.; Huang, M.; Grogan, T.; Elashoff, D.; Carpenter, C.L.; Heber, D.; Aronson, W.J. Randomized clinical trial of brewed green and black tea in men with prostate cancer prior to prostatectomy. *Prostate* **2015**, *75*, 550–559.

(35) Clifford, M. N.; Copeland, E. L.; Bloxsidge, J. P.; Mitchell, L. A. Hippuric acid as a major excretion product associated with black tea consumption. *Xenobiotica* **2000**, *30*, 317–326.

(36) Gross, G.; Jacobs, D.M.; Peters, S.; Possemiers, S.; van Duynhoven, J.; Vaughan, E.E.; van de Wiele, T. In vitro bioconversion of polyphenols from black tea and red wine/grape juice by human intestinal microbiota displays strong interindividual variability. *J. Agric. Food Chem* **2010**, *58*, 10236-10246.

(37) Van Dorsten, F.A.; Peters, S.; Gross, G.; Gomez-Roldan, V.; Klinkenberg, M.; de Vos, R.C.; Vaughan, E.E.; van Duynhoven, J.P.; Possemiers, S.; van de Wiele, T.; Jacobs, D.M. Gut microbial metabolism of polyphenols from black tea and red wine/grape juice is source-specific and colon-region dependent. *J. Agric. Food Chem* **2012**, *60*, 11331-11342.

(38) Ludwig, I. A.; Paz de Peña, M.; Cid, C.; Crozier, A. Catabolism of coffee chlorogenic acids by human colonic microbiota. *Biofactors* **2013**, *39*, 623–632.

(39) Amin, H. P.; Czank, C.; Raheem, S.; Zhang, Q.; Botting, N. P.; Cassidy, A.; Kay, C. D. Anthocyanins and their physiologically relevant metabolites alter the expression of IL-6 and

VCAM-1 in CD40L and oxidised LDL challenged vascular endothelial cells. *Mol. Nutr. Food Res*. **2015**, *59*, 1096−1106.

(40) Di Gesso, J. L.; Kerr, J. S.; Zhang, Q.; Raheem, S.; Yalamanchili, S. K.; O'Hagan, D.; Kay, C. D.; O'Connell, M. A. Flavonoid metabolites reduce necrosis factor-α secretion to a greater extent than their precursor compounds in human monocytes. *Mol. Nutr. Food Res.* **2015**, *59*, 1143−1154.

(41) Warner, E. F.; Zhang, Q.; Raheem, K. S.; O´Hagan, D.; O´Connell, M. A.; Kay, C. D. Common phenolic metabolites of flavonoids, but not their unmetabolized precursors, reduce the secretion of vascular cellular adhesion molecules by human endothelial cells. *J. Nutr*. **2016**, *146*, 465−473.

(42) Gonzalez-Sarrias, A.; Nuñez-Sánchez, M. A.; Tomás-Barberán, F. A.; Espín, J. C. Neuroprotective effects of bioavailable polyphenols-derived metabolites against oxidative stress-induced cytotoxicity in human neuroblastoma SH-SY5Y cells. *J. Agric. Food Chem*. **2017**, *6*5, 752−758.

(43) Hidalgo, M.; Martín-Santamaría, S.; Recio, I.; Sanchez-Moreno, C.; de Pascual-Teresa, B. Potential anti-inflammatory, anti-estrogenic, and angiotensin-converting enzyme inhibitory activities of anthocyanins and their gut metabolites. *Genes Nutr*. **2012**, *7*, 295−306.

(44) Forester, S. C.; Choy, Y. Y.; Waterhouse, A. L.; Oteiza, P. I. The anthocyanin metabolites gallic acid, 3-*O*-methylgallic acid, and 2,4,6-trihydroxybenzaldehyde decrease human colon cancer cell viability by regulating pro-oncogenic signals. *Mol. Carcinogen.* **2014**, *53*, 432−439.

(45) Lee, G.; Na, H. J.; Namkoong, S.; Jeong-Kowon, H.; Han, S.; Ha, K. S.; Kwon, Y. G.; Lee, H.; Kim, Y. M 4-*O*-Methylgallic acid down-regulates endothelial adhesion molecule expression by inhibiting NF-kappaB-DNA-binding activity. *Eur. J. Pharmacol*. **2006**, *55*, 143−151.

(46) Na, H. J.; Lee, G.; Oh, H. Y.; Jeon, K. S.; Kwon, H. J.; Ha, K. S.; Lee, H.; Kwon, Y. G.; Kim, Y. M. 4-*O*-Methylgallic acid supresses inflammation-associated gene expression by inhibition of redox-based NF-kappaB activation. *Int. Immunopharmacol*. **2006**, *6*, 1597−1608.

(47) Verzelloni, E.; Pellacani, C.; Tabliazucchi, D.; Tagliaferri, S.; Calani, L.; Costa, L. G.; Brighenti, F.; Borges, G.; Crozier, A.; Conte, A.; Del Rio, D. Antiglycative and neuroprotective activity of colon-derived polyphenol catabolites. *Mol. Nutr. Food Res*. **2011**, *55*, S35–S43.

FIGURE CAPTIONS

Figure 1. Structures **1**-**9**.

Figure 2. Structures **10**-**38.**

Figure 3. Theaflavin **5**, theaflavin-3-*O*-gallate **6**, theaflavin-3´-*O*-gallate **7**) and theaflavin3,3´-digallate **8** profiles of fecal fermentation samples from three volunteers over a 24 h of incubation. Data expressed as μmol, Standard error <5% of the mean in all instances (n = 3).

Figure 4. Proposed principle pathways for the catabolism of theaflavins by colonic microbiota and mammalian phase II metabolism. Red arrows indicate microbiota-mediated steps and blue arrows represent mammalian enzyme-mediated conversions. The illustrated theaflavin is theaflavin-3-*O*-gallate **6**. It assumed that the 3'-gallate **7** and the 3,3'-digallate **8** will be similarly

subject to degalloylation releasing theaflavin. Boxed names indicate the main products to accumulate in urine after theaflavin intake.

Table 1. Quantities of Individual Polyphenols in 1 g of Theaflavin Extract.[a]

Compound	Amount (μmol)	% in the theaflavin extract
Theaflavin **5**	177	17.4
Theaflavin-3-*O*-gallate **6**	307	30.4
Theaflavin-3´-*O*-gallate **7**	172	17.0
Theaflavin-3,3´-*O*-digallate **8**	332	32.8
(–)-Epicatechin **1**	0.13	0.01
(+)-Gallocatechin-3-*O*-gallate **2**	0.79	0.08
(–)-Epigallocatechin-3-*O*-gallate **3**	1.1	0.10
(–)-Epicatechin-3-*O*-gallate **4**	1.4	0.13
Procyanidin dimer B2-3-*O*-gallate **9**	22.7	2.26
Total polyphenol intake	**1014**	

[a] Standard error < 5% in all instances (n =3)

Table 2. Quantities of Structurally-Related (–)-Epicatechin Metabolites (SREMs) and Five-Carbon Side Chain Ring Fission Metabolites (5C-RFMs) Excreted in Urine 0-30 h After the Consumption of 1 g of a Theaflavin Extract by Two Volunteers.[a]

Metabolites *(number of isomers)*	**Total 0-30 h excretion**
SREMs	
(–)-Epicatechin-sulfate *(2)*	6.6 ± 3.1
(–)-Epicatechin-*O*-glucuronide	0.9 ± 0.2
O-Methyl-(–)-epicatechin-sulfate *(3)*	9.8 ± 2.5
O-Methyl-(–)-epicatechin-*O*-glucuronide	43 ± 8
Total SREMs	**60 ± 15**
5C-RFMs	
5-(4',5'-Dihydroxyphenyl)-γ-valerolactone-3'-sulfate	84 ± 3
5-(3',5'-Dihydroxyphenyl)-γ-valerolactone-4'-sulfate	256 ± 103
5-(4',5'-Dihydroxyphenyl)-γ-valerolactone-3'-*O*-glucuronide	77 ± 17
Total 5-(dihydroxyphenyl)-γ-valerolactones	**417 ± 123**
5-(4'-Hydroxyphenyl)-γ-valerolactone-3´-sulfate	158 ± 113
5-(3'-Hydroxyphenyl)-γ-valerolactone-4´-sulfate	37 ± 26
5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	12 ± 4
Total 5-(hydroxyphenyl)-γ-valerolactones	**208 ± 143**
5-(Phenyl)-γ-valerolactone-3´-sulfate	1.7 ± 0.5
Total 5-(phenyl)-γ-valerolactones	**1.7 ± 0.5**
5-(3',4'-Dihydroxyphenyl)-γ-hydroxyvaleric acid	101 ± 26
5-(Dihydroxyphenyl)-γ-hydroxyvaleric acid-sulfate	393 ± 50
5-(Dihydroxyphenyl)-γ-hydroxyvaleric acid-*O*-glucuronides *(2)*	167 ± 36
Total 5-(dihydroxyphenyl)-γ-hydroxyvaleric acids	**661± 112**
5-(3'-Hydroxyphenyl)-γ-hydroxyvaleric acid-3'-sulfate	14 ± 6
5-(4'-Hydroxyphenyl)-γ-hydroxyvaleric acid-4'-sulfate	55 ± 19
5-(4'-Hydroxyphenyl)-γ-hydroxyvaleric acid-3´-*O*-glucuronide	37 ± 14
5-(3'-Hydroxyphenyl)-γ-hydroxyvaleric acid-4´-*O*-glucuronide	12 ± 3
Total 5-(hydroxyphenyl)-γ-hydroxyvaleric acids	**118 ± 43**
5-(Phenyl)-γ-hydroxyvaleric acid-sulfate	7.2 ± 6.4
5-(Phenyl)-γ-hydroxyvaleric acid-*O*-glucuronide	24 ± 7
Total 5-(phenyl)-γ-hydroxyvaleric acids	**31 ± 14**
Total SREMS	**60 ± 15 *(1.7%)***
Total valerolactones and valeric acids	**1437 ± 436 *(5.6%)***
Total metabolites	**1495 ± 458 *(5.7%)***

[a]Data expressed as mean values in nmol ± SE (n = 2). Figure in italicised parentheses represents recovery as a percentage of intake of flavan-3-ol monomers (3.42 μmol) and procyanidin dimer (22.7 μmol).

Table 3. Quantities of Phenolic Catabolites Excreted in Urine 0-30 h After the Consumption of 1 g of a Theaflavin Extract by Two Volunteers[a].

Phenolic catabolites (*number of isomers*)		**Total 0-30 h excretion**
Phenylpropanoic acid derivatives		
Caffeic acid-3′-sulfate		0.05 ± 0.05
Ferulic acid		0.11 ± 0.07
Isoferulic acid		0.7 ± 0.1
Ferulic acid-4′-*O*-glucuronide		1.0 ± 0.6
Ferulic acid-4′-sulfate		0.9 ± 0.7
	Total	**2.8 ± 1.8**
Phenylpropionic acid derivatives		
3-(3′,4′-Dihydroxyphenyl)propionic acid **32**		0.02 ± 0.02
3-(4′-Hydroxyphenyl)propionic acid-3′-*O*-glucuronide		0.04 ± 0.01
3-(3′-Hydroxyphenyl)propionic acid-4′-sulfate		0.25 ± 0.19
3-(4′-Hydroxyphenyl)propionic acid-3′-sulfate		0.05 ± 0.01
3-(3′-Methoxyphenyl)propionic acid-4′-*O*-glucuronide		0.11 ± 0.06
3-(3′-Methoxyphenyl)propionic acid-4′-sulfate		0.10 ± 0.00
3-(4'-Hydroxyphenyl)propionic acid **10**		166 ± 91*
3-(Phenyl)propionic acid-4′-sulfate		0.05 ± 0.04
	Total	**166 ± 92**
Phenylacetic acid derivatives		
3′,4′-Dihydroxyphenylacetic acid **11**		0.38 ± 0.15*
3′-Methoxy-4′-hydroxyphenylacetic acid **12**		1.2 ± 0.2*
3′-Methoxyphenylacetic acid-4′-sulfate		0.15 ± 0.10
4’-Methoxyphenylacetic acid-3′-sulfate		0.17 ± 0.15
Methoxyphenylacetic acid-*O*-glucuronide		0.04 ± 0.00
3′-Hydroxyphenylacetic acid **13**		4.9 ± 0.6*
4′-Hydroxyphenylacetic acid **14**		6 ± 2*
Phenylacetic acid **15**		1.3 ± 1.3*
	Total	**14.1 ± 4.4***
Benzoic acid derivatives		
3,4-Dihydroxybenzoic acid **16**		0.62 ± 0.28*
3-Hydroxybenzoic acid-4-sulfate		0.33 ± 0.16
4-Hydroxybenzoic acid-3-sulfate		0.02 ± 0.02
3-Hydroxybenzoic acid **17**		0.43 ± 0.33*
4-Hydroxybenzoic acid **18**		0.6 ± 0.5*
Benzoic acid-4-sulfate **19**		3.0 ± 0.3*
Benzoic acid-3-sulfate		0.01± 0.01
3-*O*-Methylgallic acid **20**		10 ± 4*
4-*O*-Methylgallic acid **21**		27 ± 11*
Gallic acid **22**		2.3 ± 2.2*
	Total	**45 ± 19***
Hydroxycarboxylic acid derivatives		

4′-Hydroxymandelic acid		1.6 ± 0.9
3′-Methoxy-4′-hydroxymandelic acid **38**		3.8 ± 1.6
	Total	**5.4 ± 2.5**
Hydroxybenzene derivatives		
Phloroglucinol (1,3,5-trihydroxybenzene) **23**		2.7 ± 2.0*
Catechol (1,2-dihydroxybenzene) **24**		6.9 ± 2.3*
Pyrogallol (1,2,3-trihydroxybenzene) **25**		73 ± 43*
Pyrogallol-1-sulfate **26**		134 ± 50*
Pyrogallol-2-sulfate **27**		491 ± 158*
Pyrogallol-2-*O*-glucuronide **28**		14.8 ± 4.7*
	Total	**723 ± 260***
Benzoyl glycine derivatives		
3'-Hydroxyhippuric acid **29**		2.1 ± 0.4*
4'-Hydroxyhippuric acid **30**		7.3 ± 2.2*
	Total	**9.4 ± 2.6***
Significant increases in phenolic and aromatic catabolites		**955 ± 376*** ***(94%)[a]***

[a]Data corrected by subtraction of the 0-30 h baseline excretion levels and expressed as mean values in μmol ± SE (n = 2). Figure in italicised parentheses represents recovery as a percentage of 1014 μmol intake of polyphenols

*Significant higher excretion above baseline ($p<0.05$, Wilcoxon signed-rank test test)

Table 4. Total Quantities of Compounds Recovered After a 24 h In Vitro Incubation of a Theaflavin Extract with Fecal Samples from Three Donors.[a]

Compound	Donor 1 (nmol)	%	Donor 2(nmol)	%	Donor 3(nmol)	%
Theaflavins	7500	75	6700	67	6000	60
Flavan-3-ol monomers	12	36.4	11	33.3	28	84.4
5C-RFMs	17.6	6.7	25.2	9.7	104	40
Phenolic catabolites	1623	17	2530	25	100	0.1

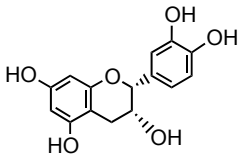
[a]Data are expressed in nmol and as percentage of the initial substrate from which they were derived Percentage recoveries based on addition to incubates of 10000 nmol of theaflavins, 33 nmol of flavan-3-ol monomers, 5C-RFMs to 260 nmol of flavan-3-ol monomers and procyanidin dimer, and phenolic catabolites to the 10000 nmol of theaflavins.

Table 5. Quantities of Compounds In Medium After 0, 1, 2, 4, 8 and 24 h Incubation of a Theaflavin Extract With Feces From Three Donors.[a]

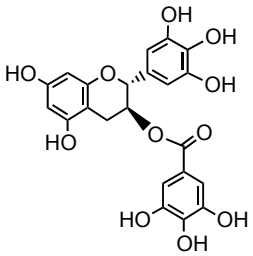
		Amount (µmol)						
Substrates	**Donor**	**0 h**	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**
Theaflavin **5**	1	1.9	2.1	2.2	2.5	3.4	3.3	6.0
	2	1.9	2.5	2.4	2.3	3.0	5.1	6.7
	3	1.9	1.9	1.8	1.9	1.8	1.8	2.1
Theaflavin-3-*O*-gallate **6**	1	4.4	4.0	4.0	3.4	3.5	3.2	1.5
	2	4.2	3.0	3.3	3.4	2.5	1.9	-
	3	4.4	4.7	4.3	3.9	2.9	2.7	2.3
Theaflavin-3´-*O*-gallate **7**	1	1.5	1.4	1.4	1.0	1.0	0.8	-
	2	1.5	1.6	0.9	0.9	0.7	0.5	-
	3	1.5	1.6	1.4	1.3	0.9	0.9	0.8
Theaflavin-3,3´-*O*-digallate **8**	1	2.5	2.4	2.2	1.8	1.3	1.2	-
	2	2.4	2.0	1.2	0.8	0.9	0.4	-
	3	2.5	2.1	12	1.7	1.0	0.8	0.8
		Amount (nmol)						
Flavan-3-ol monomers*	1	260	130	130	98	41	43	12
	2	260	154	110	87	86	72	11
	3	260	128	104	63	60	63	28
5C-RFMs								
5-(3´,4´-Dihydroxyphenyl)-γ-valerolactone **35**	1	-	-	0.07	0.09	0.06	0.1	0.1
	2	-	-	0.9	1.4	1.3	1.7	1.9
	3	-	0.3	0.7	1.2	1.4	1.7	2.3
5-(3´-Hydroxyphenyl)-γ-valerolactone **36**	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	12	11	6
5-(3´-Hydroxyphenyl)-γ-hydroxyvaleric acid **33**	1	-	-	-	-	1.0	0.7	6.5
	2	-	-	1.6	12	8	11	16

	3	-	-	6.7	63	149	128	63
	1	-	-	1.2	6.5	6.1	6.7	11
5-(Phenyl)-γ-hydroxyvaleric acid **34**	2	-	-	3.0	5.3	5.0	5.2	7.3
	3	-	4	6	22	27	29	33
3C-RFM								
	1	-	-	50	43	49	-	-
3-(3′,4′-Dihydroxyphenyl)propionic acid **32**	2	-	-	-	-	-	100	-
	3	-	-	-	90	120	200	-
1C-RFMs								
	1	-	20	80	80	50	49	-
Gallic acid **22**	2	-	40	90	140	230	170	-
	3	-	-	-	30	39	43	-
	1	-	-	51	-	-	-	-
3,4-Dihydroxybenzoic acid **16**	2	-	-	60	51	58	19	-
	3	-	-	-	-	10	10	-
	1	-	-	-	498	603	931	-
3-Hydroxybenzoic acid **17**	2		-	-	290	480	320	-
	3	-	-	-	-	-	-	-
	1	-	-	-	-	-	198	723
Benzoic acid **31**	2	-	-	1100	1050	1120	1240	982
	3	-	-	-	1230	380	1060	-
Trihydroxybenzene								
	1	-	-	90	420	530	220	900
Pyrogallol **25**	2	-	-	-	70	180	1190	1548
	3	-	-	-	-	-	-	100

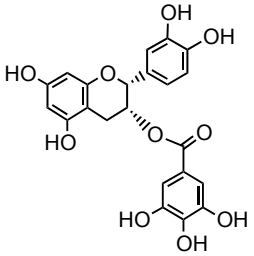
[a] Data expressed as mean values (n = 3). Standard error <15% of mean values. Structurally-related (–)-epicatechin metabolites, SREMs; 5C-side chain ring fission metabolites, 5C-RFMs; 3 carbon side chain ring fission metabolite, 3C-RFM; 1-C side chain ring fission metabolites, 1C-RFMs



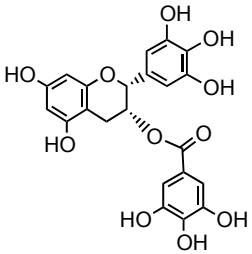
(–)-Epicatechin **1**



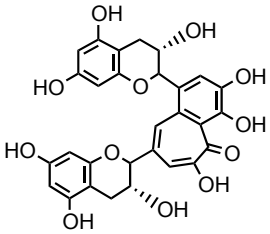
(+)-Gallocatechin-3-*O*-gallate **2**



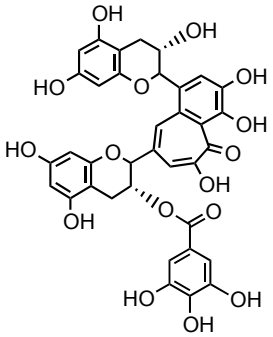
(–)-Epicatechin-3-*O*-gallate **3**



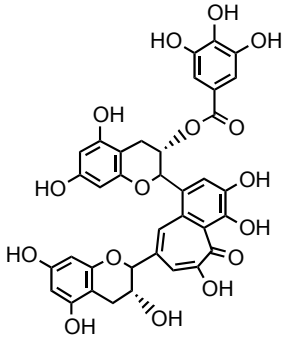
(–)-Epigallocatechin-3-*O*-gallate **4**



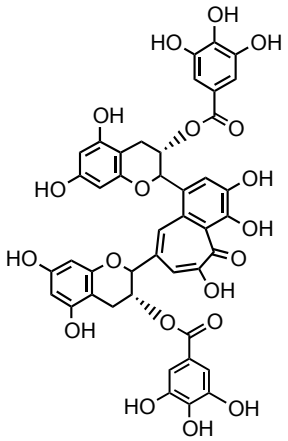
Theaflavin **5**



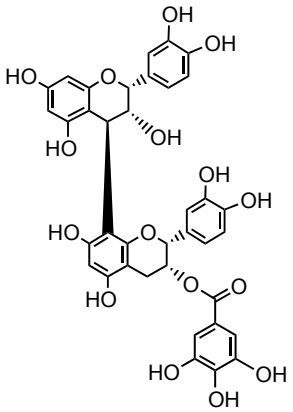
Theaflavin-3-*O*-gallate **6**



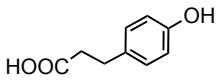
Theaflavin-3'-*O*-gallate **7**



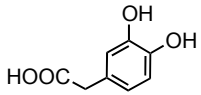
Theaflavin-3,3'-*O*-digallate **8**



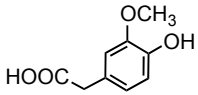
Proanthocyanidin dimer B_2 dimer-3'-*O*-gallate **9**



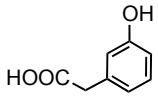
3-(4'-Hydroxyphenyl)propionic acid **10**



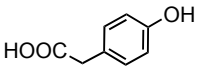
3',4'-Dihydroxyphenylacetic acid **11**



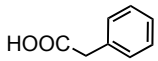
3'-Methoxy-4'-hydroxyphenylacetic acid **12**



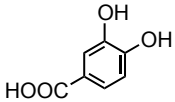
3'-Hydroxyphenylacetic acid **13**



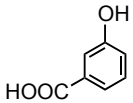
4'-Hydroxyphenylacetic acid **14**



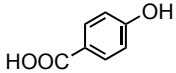
Phenylacetic acid **15**



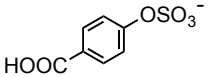
3,4-Dihydroxybenzoic acid **16**



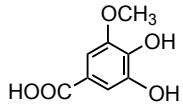
3-Hydroxybenzoic acid **17**



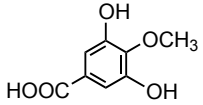
4-Hydroxybenzoic acid **18**



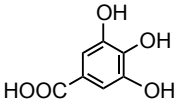
Benzoic acid-4-sulfate **19**



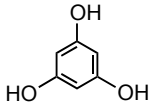
3-*O*-Methylgallic acid **20**



4-*O*-Methylgallic acid **21**

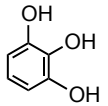


Gallic acid **22**

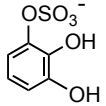


Phloroglucinol **23**

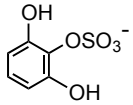
Catechol **24**



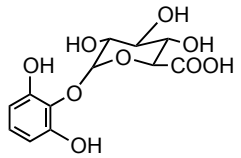
Pyrogallol **25**



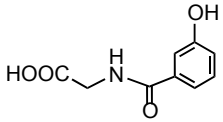
Pyrogallol-1-sulfate **26**



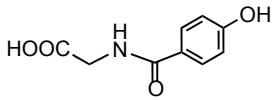
Pyrogallol-2-sulfate **27**



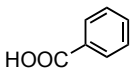
Pyrogallol-2-*O*-glucuronide **28**



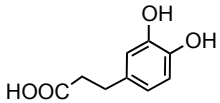
3'-Hydroxyhippuric acid **29**



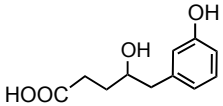
4'-Hydroxyhippuric acid **30**



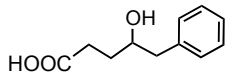
Benzoic acid **31**



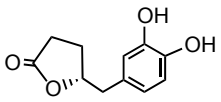
3-(3,4'-Dihydroxyphenyl)propionic acid **32**



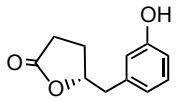
5-(3'-Hydroxyphenyl)-γ-hydroxyvaleric acid **33**



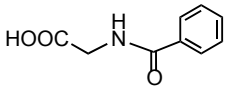
5-(Phenyl)-γ-hydroxyvaleric acid **34**



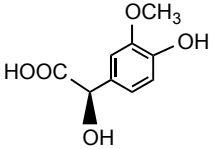
5-(3',4'-Dihydroxyphenyl)-γ-valerolactone **35**



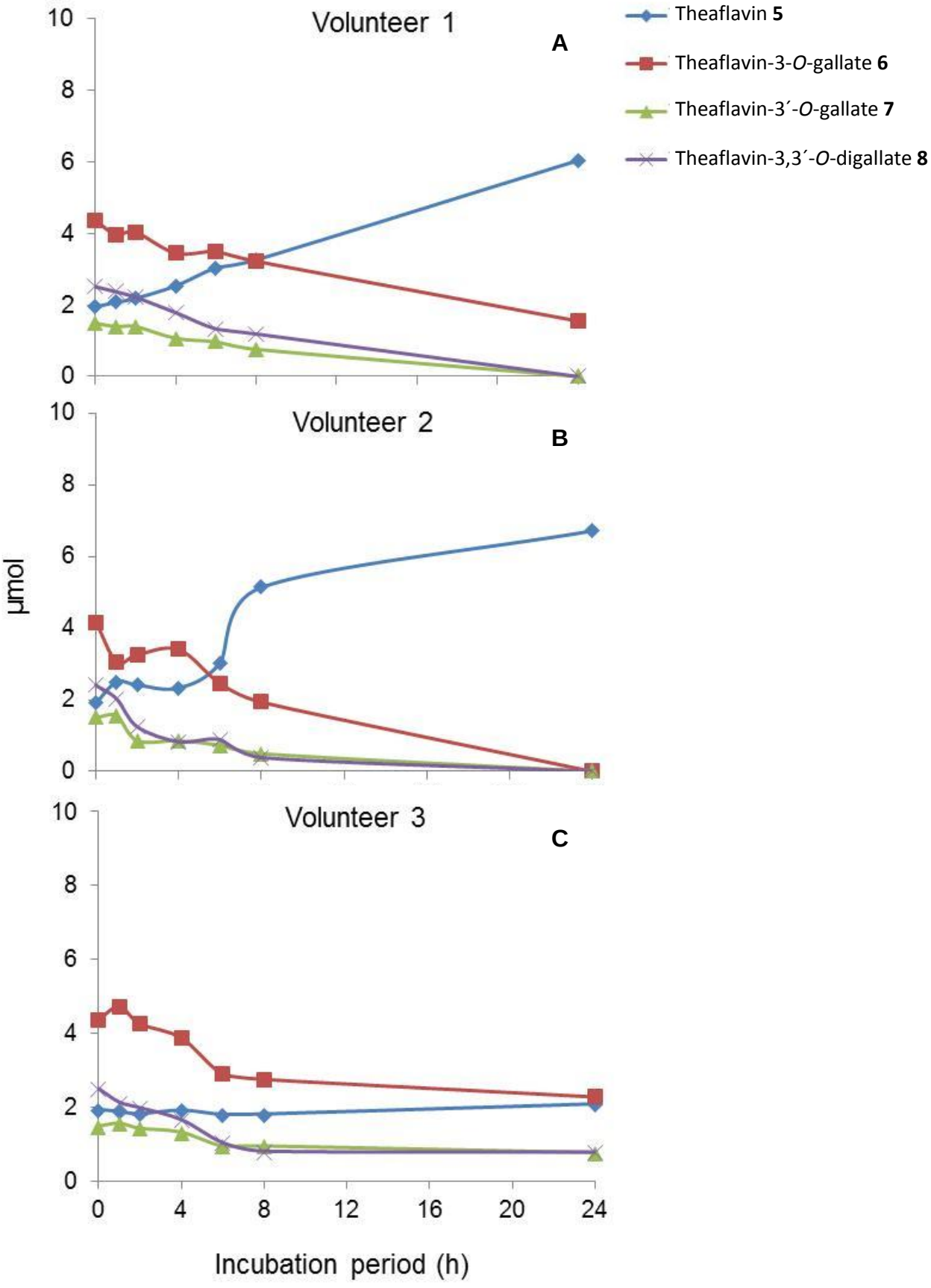
5-(3'-Hydroxyphenyl)-γ-valerolactone **36**

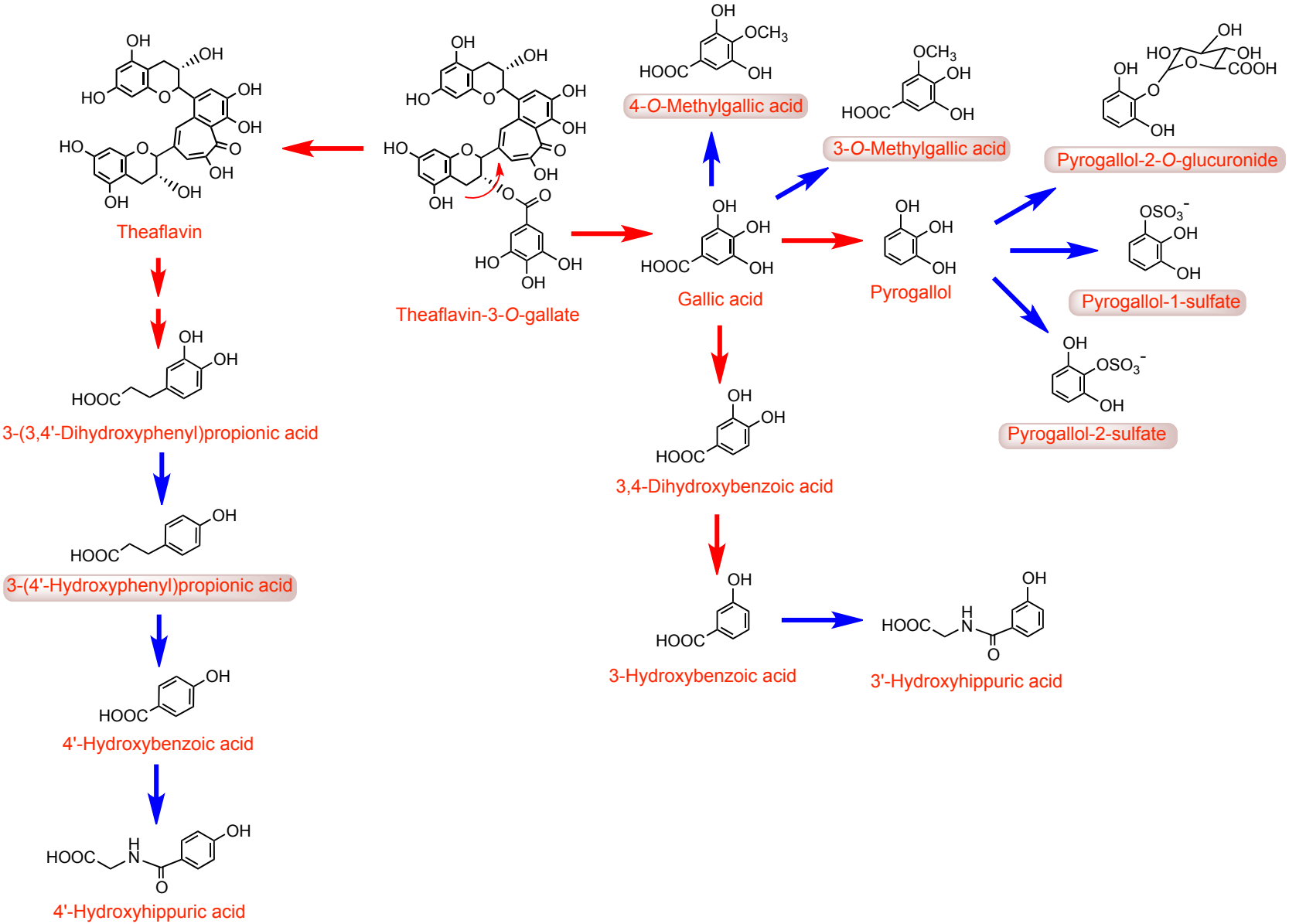


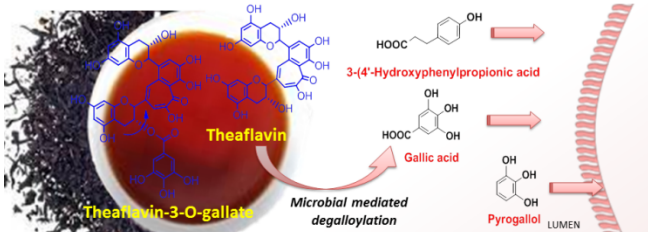
Hippuric acid **37**



3'-Methoxy-4'-hydroxymandelic acid **38**


Volunteer 1
A
Volunteer 2
B
Volunteer 3
C
µmol
Incubation period (h)
Theaflavin 5
Theaflavin-3-O-gallate 6
Theaflavin-3´-O-gallate 7
Theaflavin-3,3´-O-digallate 8


Theaflavin
Theaflavin-3-O-gallate
4-O-Methylgallic acid
3-O-Methylgallic acid
Pyrogallol-2-O-glucuronide
Gallic acid
Pyrogallol
Pyrogallol-1-sulfate
Pyrogallol-2-sulfate
3,4-Dihydroxybenzoic acid
3-Hydroxybenzoic acid
3'-Hydroxyhippuric acid
3-(3,4'-Dihydroxyphenyl)propionic acid
3-(4'-Hydroxyphenyl)propionic acid
4'-Hydroxybenzoic acid
4'-Hydroxyhippuric acid


Theaflavin
Theaflavin-3-O-gallate
Microbial mediated degalloylation
3-(4'-Hydroxyphenylpropionic acid
Gallic acid
Pyrogallol
LUMEN